PLEISTOCENE INFRASPECIFIC EVOLUTION IN *JUNIPERUS ASHEI* BUCH.

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ABSTRACT

The Pleistocene and recent distributions of the taxa are discussed. A new variety of *Juniperus ashei*, *J. ashei* Buch. var. *ovata* R. P. Adams is recognized from west Texas and northern Mexico. It differs from typical *J. ashei* in having whip leaf glands that are oval to elongate rather than hemispherical, smaller female cones, smaller seeds and more seeds per cone. In addition, the leaf oil of var. *ovata* is higher in α-pinene, myrcene, limonene, γ-terpinene, bornyl acetate and elemol but lower in linalool, trans-sabinene hydrate, trans-p-menth-2-en-1-ol, camphor, trans carveol and carvone. RAPDs analysis revealed that the varieties are distinct in their DNA. It is hypothesized that var. *ovata* is a pre-Pleistocene relict, while var. *ashei* is a recently (late Pleistocene - Holocene) derived taxon.

KEY WORDS: *Juniperus, J. ashei* var. *ovata,* RAPDs, essential oils, Pleistocene distributions, Cupressaceae

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Juniperus ashei is a small tree that grows abundantly on limestone on the Edwards plateau in central Texas with disjunct populations on limestone in Arkansas, Missouri, and Oklahoma as well in Coahuila, Mexico (Fig. 1). The Edwards Plateau (limestone) region of central Texas supports dense populations covering millions of acres, whereas the disjunct populations (Fig. 1) often have almost pure stands of *J. ashei*, that may cover only a few acres.

Studies of geographic variation in *Juniperus ashei* have shown that the species has divergent populations in the semi-arid margins of its

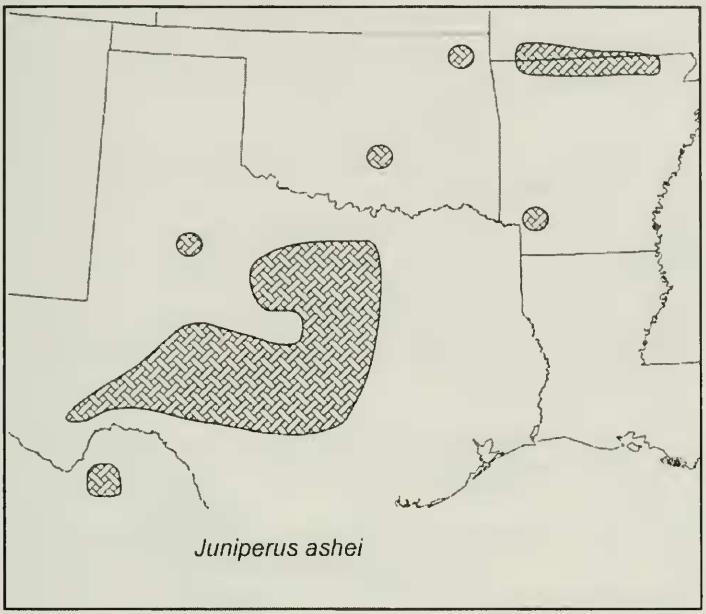

Figure 1. Distribution of *J. ashei*, adapted from Adams (2004). Note the disjunct populations.

range (Adams, 1977, 2004). Both leaf terpenoids and morphology were subjected to canonical variate analyses (Adams, 1977). These coordinate scores, when plotted onto maps, show a sharp divergence in Mexico and west Texas (popns. 12, 13, 26) from the central Texas and northward populations, in both the terpenes and morphology (Fig. 2). Notice that populations 12 (Ozona, TX), 13 (Comstock, TX), 25 (Pandale, TX) and 26 (Coahuila, MX) are divergent in both their essential oils (Fig. 2, left) and morphology (Fig. 2, right). In addition, population 17 (nw of New Braunfels, TX) is somewhat divergent in its essential oil, but less so in its morphology (Fig. 2, right). It is interesting to note that all the other populations of *J. ashei*, even those in isolated areas, show almost no variation in either the leaf oils or the morphology. The Trans-Pecos, Texas and Mexico populations

(ancestral or pre-Pleistocene) have more elliptical whip leaf glands, whereas the balance of *J. ashei* populations (Holocene or post-

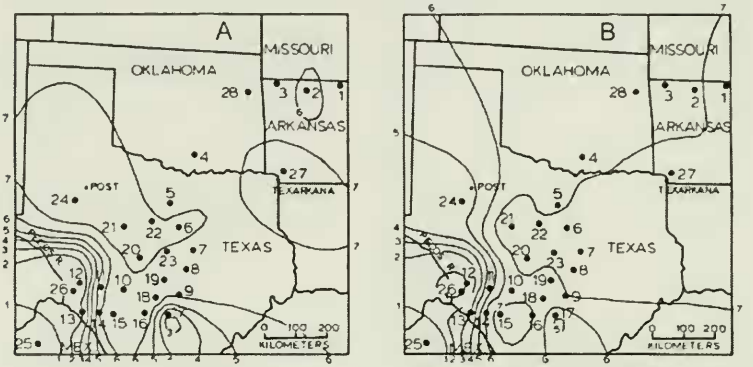

Figure 2. Principal coordinate analyses (PCO) of terpenoids (A) and morphology (B) of *J. ashei*. Adapted from Adams, 1977.

Pleistocene) have hemispherical glands that are unique to *Juniperus* (Adams, 2004). The hemispherical glands seem to be a derived condition in *Juniperus*.

Although there is considerable evidence of a continuous band of sclerophyllous vegetation from central Texas into northern Mexico during the Tertiary (Axelrod, 1975), it is more productive to focus on events in the Pleistocene, particularly the last pluvial and interglacial periods. According to King (1973), the western Ozarks were covered with boreal spruce forest from about 25,000 to at least 13,000 B.P., with pine parkland preceding the boreal spruce forest. The pine parkland and boreal spruce forest both appeared to have been pushed southward from the north (Dillon, 1956). Figure 3 shows the hypothetical vegetation during the pluvial period (modified from Adams, 1977). The area south of the Ozarks may have been pine woodland or parkland (see Bryant, 1969). A pine-spruce woodland seems likely on the Llano Estacado of northwest Texas according to Hafsten (1961). Bryant (1969) suggested that, based on pollen profiles, the present Chihuahuan desert area around Del Rio, TX (430 m) was pinyon woodland. Wells (1966), using data obtained from rat middens from the Big Bend region of Texas, concluded that life zones

descended about 800 m for pinyon-juniper (*J. pinchotii* in that case), allowing the advance of pinyon-juniper into most of the present desert region between the Big Bend and

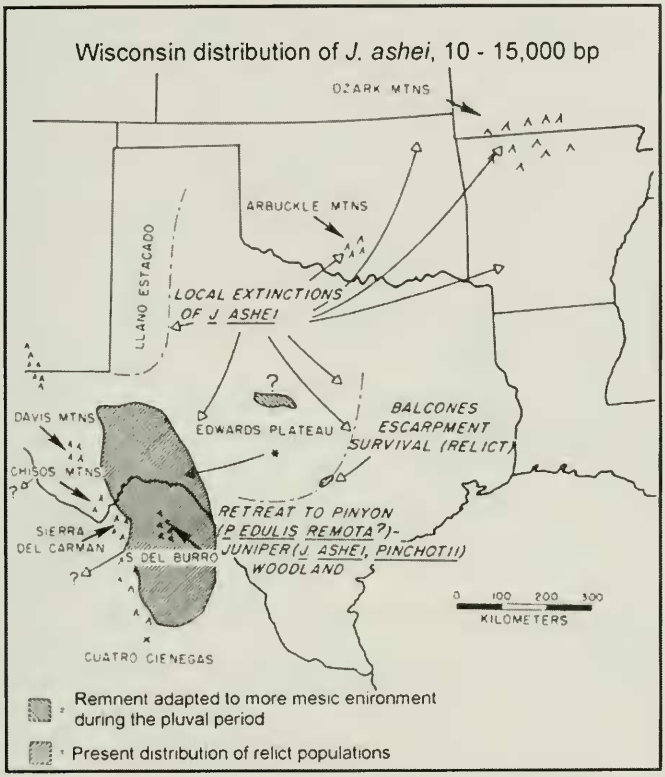

Figure 3. Possible *J. ashei* distribution during the Wisconsin era (from Adams, 1977, 2004).

Del Rio. Typical *J. pinchotii*, and the ancestral (Pleistocene) type, *J. ashei,* have been found growing just south of the Sierra de Carmen mountains of the Big Bend region (Adams, 2004). It appears that the Sierranas del Burro, Mexico may have been an important refugium or "island point" in the pinyon-juniper woodland. A mixed deciduous woodland with conifers is postulated in central Texas (Bryant, 1969) based on pollen profiles.

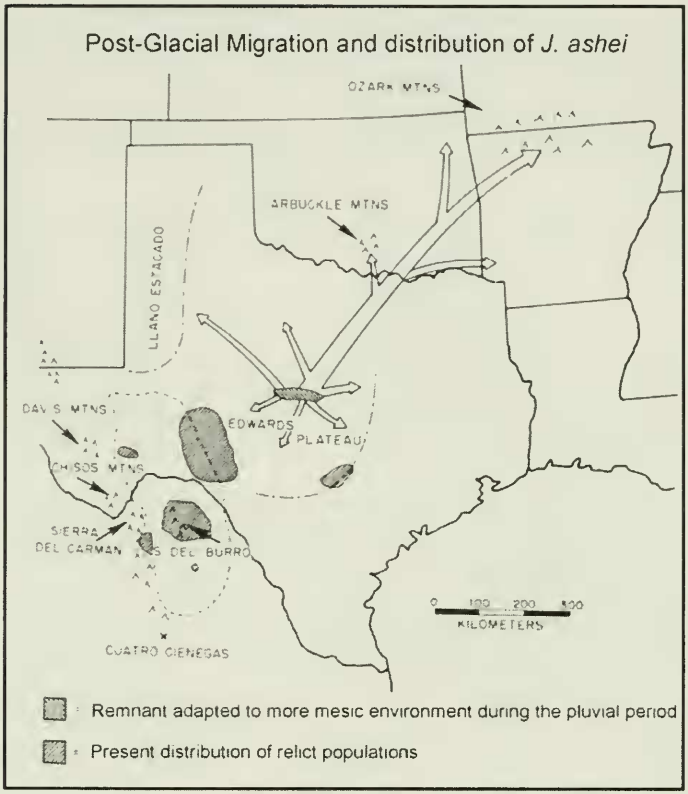

Figure 4. Postulated post-glacial recolonization of *J. ashei* onto limestone producing very uniform populations (Adams, 1977, 2004).

At the end of the Wisconsin glacial advance (10,000 - 13,000 yr bp), the central Texas, Oklahoma and Arkansas populations of *J. ashei* were likely extinct because this area was a much wetter and cooler spruce woodland (Fig. 3).

Adams (1977, 2004) postulated that *J. ashei* was pushed south into refugia in west Texas and Mexico (Fig. 3). It appears that the ancestral (Pleistocene) *J. ashei* was lower in camphor and had somewhat elongated whip leaf glands. The more recently derived (Holocene) populations (Fig. 4) are higher in camphor and have whip leaves with

hemispherical oil glands that are unique within *Juniperus* (Adams, 2004).

During the same period, *J. ashei* may have expanded south and west into the Chihuahuan desert (Wells, 1966), but not as far south as Cuatro Cienegas, Coahuila, Mexico (Meyer, 1973). Migration of populations to regions west of the Sierra del Carmen was also possible because *J. ashei* grows at the top of La Cuesta pass just south of the Sierra del Carmen (Adams, 1977). With this model, populations of *J. ashei* were forced to extinction in central Texas, Oklahoma, Arkansas, and Missouri. The subsequent recolonization in the Holocene could then take place as depicted in figure 4, over a very short time from a relictual population in central Texas that may have gone through a selection 'bottleneck' perhaps coupled with genetic drift. This 'relict' population would have had considerably more camphor in the oil, more hemispherical glands, larger female cones, fewer seeds (therefore a higher pulp to seed ratio as a reward for birds), and a more lax foliage which seems to be associated with more mesic junipers. The rapid colonization of limestone outcrops (Fig. 4) could then lead to a uniform taxon from central Texas to the Ozarks. In addition, this Holocene type of *J. ashei*, was competitive in invading grasslands that still flourished in the post-Wisconsin pluvial period (Adams et al. 1998a). The pre-Pleistocene (ancestral) *J. ashei* is restricted to drier, rocky habitat in far west Texas and northern Mexico.

The purpose of this paper is to present new analyses of the leaf oils and DNA fingerprinting (RAPDs) of the wide-spread *J. ashei* from the Edwards Plateau, and *J. ashei* from the semi-arid area around Ozona, TX, and to re-evaluate the taxonomic status of the divergent, pre-Pleistocene populations.

MATERIALS AND METHODS

Specimens used in this study: *Juniperus ashei,* Coryell Co., TX, *Adams 7424-42* 2 km se of jct. of CR314 and FR107 on CR314; Crockett Co., TX, *Adams 7463-82*, 5 km w of Ozona, on US290. Voucher specimens are deposited at Baylor University (BAYLU).

Fresh leaves (200 g. fresh wt.) were steam distilled for 2 h using a circulatory Clevenger apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored

at -20°C until analyzed. The extracted leaves were oven dried (48h, 100°C) for determination of their oil yields.

The essential oils were analyzed on a HP5971 MSD mass spectrometer, directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2006 for operating details). Identifications were made by library searches of our volatile oil library (Adams, 2006), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by TIC.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). The RAPD analyses follow that of Adams and Demeke (1993). Ten-mer primers were purchased from the University of British Colombia (5'-3'): 134, AAC ACA CGA G; 153, GAG TCA CGA G; 204, TTC GGG CCG T; 218, CTC AGC CCA G; 227, CTA GAG GTC C; 236, ATC GTA CGT G; 239, CTG AAG CGG A; 244, CAG CCA ACC G; 250, CGA CAG TCC C; 265, CAG CTG TTC A; 268, AGG CCG CTT A; 338, CTG TGG CGG T; 346, TAG GCG AAC G; 347, TTG CTT GGC G.

PCR stock solutions (Taq, primer, buffer) were made in bulk so that all the PCR reaction tubes for a primer were prepared using the same bulk stock. This is a critical factor for minimizing variation in band intensities from sample to sample (see Adams et al. 1998a, for protocols to minimize PCR band variation). PCR was performed in a volume of 15 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM $MgCl_2$, and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 µM primers, 0.3 ng genomic DNA, 15 ng BSA and 0.6 unit of Taq DNA polymerase (Promega). A negative control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination. DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). Samples were run in duplicate to insure reproducibility (Adams et al. 1998a). A temperature profile was obtained for each well of the thermocycler to be sure that no variation existed among wells in the heating/ cooling block. The thermal cycle used was: 94°C (1.5 min) for initial strand separation, then 40 cycles of 40°C (2 min), 72°C (2 min), 91°C (1

min). Two additional steps were used: 40°C (2 min) and 72°C (5 min) for final extension. The temperature inside a PCR tube containing 15 µl buffer was monitored with a temperature probe, quantitated and printed for each step for each of the 40 cycles for every PCR run (Adams et al.1998a) to insure that each cycle met temperature specifications and that each PCR run was exactly the same. Amplification products were analyzed by electrophoresis on 1.5% agarose gels, 75V, 55 min, and detected by staining with ethidium bromide. The gels were photographed over UV light using Polaroid film 667 and scanned to digital images. The digital images were size normalized in reference to pGem® DNA size markers before band scoring. Bands were scored as present (1) and absent (0). Bands that were inconsistent in replicate analyses were not scored.

Associational measures were computed using absolute character state differences (Manhattan metric), divided by the maximum observed value for that character over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principle coordinate analysis (PCO) was performed by factoring the associational matrix using the formulation of Gower (1966) and Veldman (1967). It should be noted that problems of homology of RAPD DNA bands on agarose gels can be significant (Rieseberg, 1996), but these errors can be accounted for using multivariate statistical methods (PCO) (see Adams and Rieseberg, 1998). A minimum spanning diagram was constructed by selecting the nearest neighbor for each taxon from the pair-wise similarity matrix, then connecting those nearest neighbors as nodes (Adams et al. 2003).

RESULTS AND DISCUSSION

Comparing the leaf essential oils semi-arid (ancestral) versus more mesic, Edwards plateau junipers revealed that they differ mostly in a quantitative fashion (Table 2). Camphor is considerably larger (Table 2) in more recent populations (69.1%) than in ancestral populations (53.5%). In contrast, bornyl acetate is must larger in ancestral (15.6%) than in recent (6.3%) populations (Table 2). Four (non-trace) compounds differ qualitatively: trans-sabinene hydrate, trans-p-menth-2-en-1-ol, verbenone, and sandaracopimara-8(14),15-diene (Table 2). Several other compounds differ quantitatively: α-pinene, myrcene, p-

cymene, limonene, γ-terpinene, linalool, trans carveol, carvone and elemol (Table 2).

Principal Coordinate Analysis (PCO) using 175 RAPD bands resulted in the complete separation of the ancestral (Ozona) from the recent (Waco, Edwards plateau) plants (Fig. 5). The first coordinate

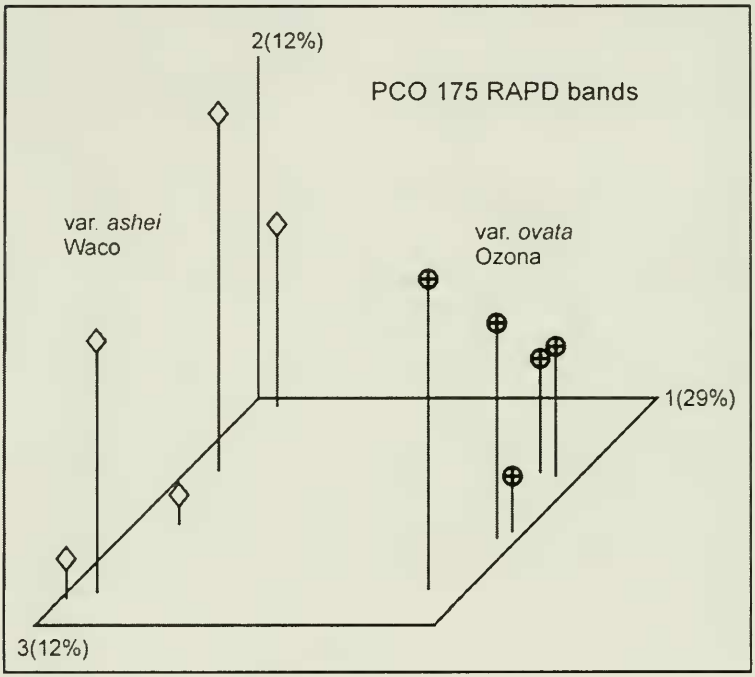

Figure 5. PCO of *J. ashei* based on 175 RAPD bands.

removed 29% of the variance among individuals and clearly shows that the populations are distinct in their DNA.

Type material of *J. ashei* consisted of one male and three female specimens (Hall, 1954). Because of this, Hall (1954) selected a female specimen (acc. number *22520*, 16 Sept. 1923, UNC) and designated it as the lectotype. All of the material cited by Buchholz (1930) was collected on limestone bluffs, above the White River, near Sylamore, Arkansas. It is clear from the figure in Buchholz (fig. 1,) that his

specimens had hemispherical glands on the whip leaves were representative of the post-Pleistocene *J. ashei.*

Based on the differences in morphology, leaf essential oils and DNA, there is sufficient differentiation to recognize the ancestral, pre-Pleistocene populations from far west Texas and Coahuila, Mexico as a distinct variety:

Juniperus ashei var. ***ovata*** R. P. Adams, **var. nov**. TYPE: U. S. A., Texas, Crockett Co., 5 km w. Ozona, 6 Dec. 1994, *R. P. Adams 7463* (HOLOTYPE: BAYLU, PARATYPES: *R. P. ADAMS 7664, 7465, 7466, 7467* (BAYLU).

Junipero ashei var. ashei similis sed differt flagellifoliis glandibus elevatis ovalibus vel elongatis, strobilis minoribus, et seminibus in quoque strobilo plus numerosis.

The new variety is like *Juniperus ashei* var. *ashei*, but instead of hemispherical glands, the glands are oval to elongated on the whip leaves. *Juniperus ashei* var. *ovata* also has smaller cones, and more seeds per cone than *J. a.* var. *ashei.*

Other specimens examined: MEXICO, Coahuila, *Adams 1066-1076.* U.S.A., Texas, Crockett Co., Ozona, *Adams 7424-42* (BAYLU), Coryell Co., TX, *Adams 7463-82* (BAYLU!).

Key to *J. ashei* varieties:

1. Glands on whip leaves hemispheric; female cones (8) 9 (10) mm in diameter; seeds 16-27 mm^2, 1 (rarely 2, avg. 1.01) per cone .....*J. ashei* var. *ashei*
1. Glands on whip leaves oval to elliptical; female cones (5) 6 (8) mm diam.; seeds 13-16 mm^2, 2 (avg. 1.7), per cone .....*J. ashei* var. *ovata*

The whip leaf glands are illustrated in figure 5. Notice hemispherical glands on var. *ashei* (Fig. 6, left) and the raised, oval to elongated glands on var. *ovata* (Fig. 6, right). It should be noted that a few nearly hemispherical glands are present on whip leaves of var. *ovata.* This is informative, as these characters can be used to distinguish var. *ovata* from var. *ashei*, yet exclude other nearby juniper

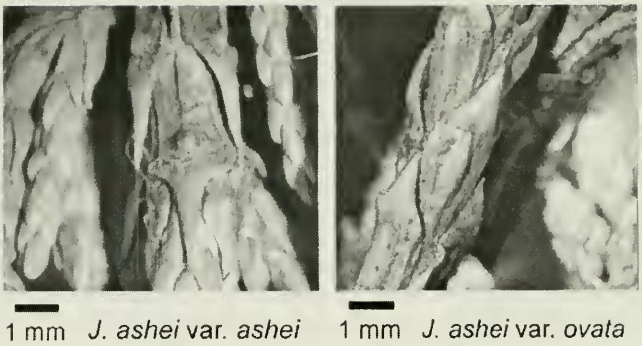

Figure 6. Comparison of whip leaf glands for *J. ashei* var. *ashei* and *J. a.* var. *ovata*.

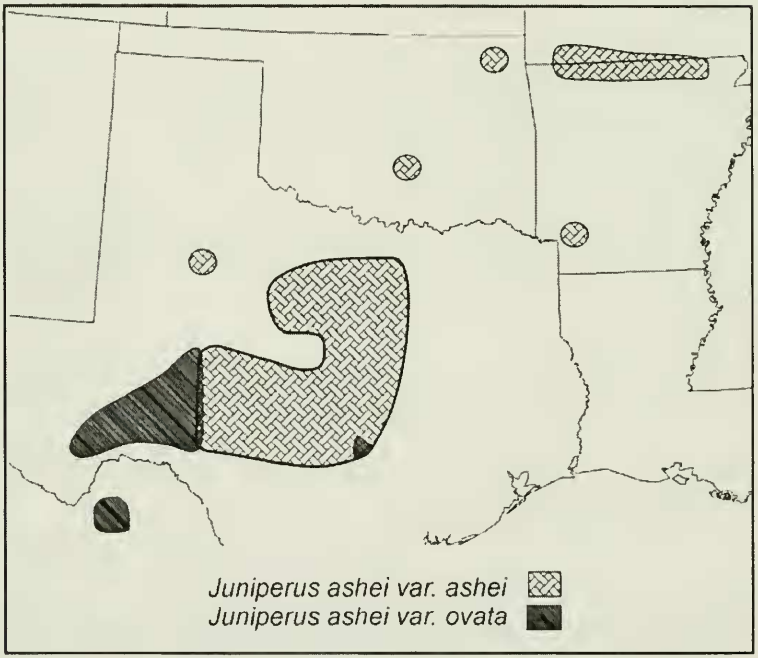

Figure 7. Distribution of *J. ashei* var. *ashei* and *J. a.* var. *ovata*.

species such as *J. monosperma* (Englem.) Sarg. *J. pinchotii* Sudw. and *J. coahuilensis*. (Mart.) Gaussen ex R. P. Adams.

The distribution of the two varieties is shown in fig. 7. The area of possible sympatry in west Texas and around New Braunfels is not well understood and additional field collections are needed to define better their distributions in these areas.

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Table 1. Differences between *J. ashei* var. *ashei* (recent, post-Pleistocene) and *J. a.* var. *ovata* (ancestral, Pleistocene relicts).

Character	*J. a.* var. *ashei*	*J. a.* var. *ovata*
female cone diameter	larger (8)9(10) mm	smaller (5)6(8)mm
seeds per cone	fewer (1.01)	more (1.7)
seed size (L x W)	larger (16-27 mm^2)	smaller (13-16 mm^2)
whip leaf gland length/ sheath length	smaller ratio (0.20-0.30)	larger ratio (> 0.40)
whip leaf gland shape	hemispherical (1.0 - 1.5)	raised, oval to ellipse (2.0 - 2.5)
branching angle	narrow (45 - 40°)	wider (45 - 55°)

Table 2. Comparisons of the per cent total oil for the leaf essential oils of *J. ashei* var. *ashei* and *J. a.* var. *ovata*. Large differences in concentrations are highlighted in boldface.

RI	Compound	var. *ashei*	var. *ovata*
921	tricyclene	1.3	1.1
933	**α-pinene**	**0.4**	**3.8**
946	camphene	1.6	1.6
969	sabinene	t	0.3
974	β-pinene	t	-
988	**myrcene**	**0.5**	**2.6**
1001	δ-2-carene	t	-
1002	α-phellandrene	t	t
1008	δ-3-carene	t	0.1
1014	α-terpinene	t	t
1020	**p-cymene**	**2.0**	**0.7**
1024	**limonene**	**3.5**	**7.7**
1025	β-phellandrene	t	t
1054	**γ-terpinene**	**0.2**	**0.8**
1067	cis-linalool oxide (furanoid)	t	-
1078	camphenilone	t	-
1084	trans-linalool oxide (furanoid)	0.3	0.4
1086	terpinolene	t	t
1095	**linalool**	**1.4**	**0.4**
1098	**trans-sabinene hydrate**	**0.2**	**-**
1100	isopentyl 2-methyl butanoate	t	-
1112	3-methyl butanoate, 3-methyl-3-butenyl-	t	t
1118	cis-p-menth-2-en-1-ol	t	t
1122	α-campholenal	t	-
1136	**trans-p-menth-2-en-1-ol**	**0.2**	**-**
1141	**camphor**	**69.1**	**53.3**
1145	camphene hydrate	0.3	0.3
1165	borneol	2.2	2.8
1174	terpinen-4-ol	0.3	0.5
1179	p-cymen-8-ol	0.3	0.1
1186	α-terpineol	0.1	t

Table 2, continued.

RI	Compound	var. *ashei*	var. *ovata*
1204	**verbenone**	**0.1**	-
1207	trans-piperitol	0.2	t
1215	**trans-carveol**	**0.7**	**t**
1218	endo-fenchyl acetate	t	-
1226	cis-carveol	t	t
1239	**carvone**	**0.8**	**t**
1249	piperitone	t	-
1273	trans-carvone oxide	t	-
1287	**bornyl acetate**	**6.3**	**15.6**
1289	p-cymen-7-ol	t	-
1298	carvacrol	t	-
1339	trans-carvyl acetate	t	t
1340	piperitenone	t	-
1548	**elemol**	**0.2**	**0.9**
1649	β-eudesmol	t	0.4
1652	α-eudesmol	t	0.5
1968	**sandaracopimara-8(14),15 -diene**	**0.2**	**-**
1987	manoyl oxide	3.6	3.2
2055	abietatriene	0.2	0.2
2087	abietadiene	0.2	0.3
2282	sempervirol	1.1	0.5
2314	trans-totarol	0.7	0.3
2331	trans-ferruginol	0.2	0.1

0.05% are denoted as traces (t). Unidentified components less than 0.5% are not reported. RI is the arithmetic retention index in DB-5.